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DEVELOPMENT AND VALIDATION OF A COMPUTATIONAL MODEL FOR INTRA-CELLULAR CIRCADIAN OSCILLATORS

Brigham and Women's Hospital

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13. ABSTRACT ***(Maximum 200 Words)***

Circadian rhythms are endogenous rhythms in physiology or behavior with a cycle length near 24 hours. Circadian rhythms are relevant to military activities because the ability of military personnel to maintain high level of cognitive performance and vigilance for long intervals is degraded by human factors, including fatigue, sleep loss and other circadian factors. Underlying circadian rhythmicity is a molecular feedback loop. This project developed mathematical models of the mammalian circadian oscillator. Our approach was to gather information on parameters needed for model development through biochemical experiments, develop mathematical models of the mammalian circadian oscillator, and experimentally test predictions from these models. We created a reduced molecular model, with the long-tem objective of incorporating a molecular model into an existing model of the influence of light and rhythmicity on human performance. Our experimental and modeling efforts led to several publications and the contribution of software for numerous circadian models to BioSpice. Understanding of the circadian oscillatory mechanism has the potential for new strategies in resetting the circadian clock to promote alertness and to enhance physiological synchronization to new environments after transmeridian travel. Our development of detailed molecular models of the mammalian clock provides an important first step towards these objectives.

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PREFACE: Sites and Participants

Award PI and Site Heads

The award under this contract was made to Dr. Megan Jewett, head of the Biomathematical Modeling Unit, Brigham & Women's Hospital (BWH) and Harvard Medical School, Boston. Subcontracts were made to the Courant Institute of Mathematical Sciences, New York University (subcontract PI/site head: Dr. Charles Peskin) and to the University of Massachusetts Medical School (subcontract PI/site head: David Weaver). Drs. Leloup and Goldbeter of Brussels were engaged as consultants.

Dr. Jewett went on medical leave of absence in March 2003. Dr. Weaver succeeded Dr. Jewett as interim PI (March-Sept 2003) and then as PI in September 2003. The prime award remained at BWH despite the change of PI. Dr. Elizabeth Kllerman succeeded Dr. Jewett as the site head for the effort at Brigham & Women's Hospital.

This report, by David Weaver, is the final report for all activities under this award.

Team Organization and Personnel

Our Team's efforts were organized into three components:

- a Modeling Group (led by Dr. Richard Kronauer),
- an Experimental Group (led by Dr. David Weaver), and
- a Software Group (led by Mr. Dennis Dean).

The personnel contributing to the Modeling Group for this project were Megan Jewett, Richard Kronauer, Premananda Indic, Melissa St. Hillarie and Elizabeth Klerman (of the Biomathematical Modeling Unit at Brigham & Women's Hospital), Daniel Forger and Charles Peskin (of the Courant Institute/NYU), and Jean-Christophe LeLoup and Albert Goldbeter (of University Libre de Brussels, Belgium).

The personnel of the Experimental Group were David Weaver, Choogon Lee, Charlotte von Gall, Otto Gildemeister, Tony Gotter, and Christopher Lambert, all of UMass Medical School, Worcester.

The personnel of the Software Group were Dennis Dean and Katherine Gurdziel of the Biomathematical Modeling Unit at Brigham & Women's Hospital.

SUMMARY

The understanding of circadian rhythms is relevant to military activities because the ability of military personnel to maintain a high level of cognitive performance and vigilance for long intervals is degraded by human factors, of which fatigue, sleep loss and circadian factors are major components. Circadian rhythms are endogenous rhythms with a cycle length of approximately 24 hours. The molecular mechanism underlying these physiological and behavioral rhythms is an intracellular molecular feedback loop, based on the rhythmic production of specific proteins within pacemaker structures. Prior work on mathematical modeling of molecular circadian oscillators focused on the fruit fly, *Drosophila melanogaster*. The major focus of this project, supported by AFRL/DARPA, was to generate mathematical models of the mammalian circadian oscillator. Our approach was to gather information on the parameters needed for model development through experiments, develop mathematical models of the mammalian circadian oscillator, and generate and experimentally test predictions from these models. A second level of effort was to mathematically simplify the complex mathematical models to generate a reduced molecular model, with the long-term objective of incorporating a molecular model into an existing model of the influence of light and rhythmicity on human performance. Our experimental and modeling efforts led to publication of several papers, including two new mathematical models of the mammalian circadian oscillator. An additional, major contribution of our effort was the development and contribution of software for numerous circadian models to BioSpice, allowing these models to become accessible to the general modeling community. An increased understanding of the circadian clock has the potential to lead to new strategies for resetting the circadian clock, promoting alertness and enhancing physiological synchronization to new environments after trans-meridian travel. Our development of detailed molecular models of the mammalian circadian clock provides an important first step toward these objectives.

INTRODUCTION

The mammalian circadian timing system is responsible for the regulation of 24-hour rhythmicity in many physiological functions. The objective of this Project was to understand this system biologically, and to model it mathematically, leading to the generation of experimentally

testable hypotheses and the potential for development of strategies to manipulate the circadian pacemaker. Our work focuses on the 24-hour biochemical oscillations that occur within individual cells. The suprachiasmatic nuclei (SCN) of the anterior hypothalamus contain the master circadian pacemaker in mammals. Long-term recordings of electrical activity rhythms from SCN neurons indicate that individual cells can maintain circadian timekeeping (Welsh et al., 1995; Liu et al., 1997). Furthermore, a mutation that influences rhythmicity in the whole animal has a corresponding effect on individual "clock cells" in vitro (Liu et al., 1997). Thus, in terms of fundamental brain mechanisms, the circadian system is among the most tractable models for providing a complete understanding of the cellular and molecular events connecting genes to behavior. A second focus of investigation is identification of the biological mechanisms by which light influences the mammalian circadian clock, and incorporation of the influence of light into our mathematic models. It is important to define the influence of light biologically and in the molecular models, as a model already exists describing the influence of light and rhythmicity on human performance.

Physiology of Circadian Rhythms

The most widely appreciated daily rhythm in humans is the sleep-wake cycle, but numerous other aspects of physiology and behavior also vary over the course of the 24-hour day. Alertness, sleep latency, hormone levels, and other functions are rhythmic, with a cycle length of approximately (circa) one day (dias, thus the term circadian), even in constant environmental conditions. These endogenous rhythms are generated by a circadian timing system. This system can be thought of as consisting of a pacemaker (or clock), along with the input pathways that allow information to reach it, and output pathways that lead to overt expression of rhythms in physiology and behavior. Many rhythms persist in the absence of rhythmic environmental cycles, showing that they are not simply a response to a varying environment and thus can be called circadian rhythms, reflecting that they are endogenous in nature. An important feature of the circadian timing system is that the clock mechanism can be reset by environmental stimuli. Light is the most effective agent for synchronizing the circadian clock to the 24-hour day in most species.

Cellular Circadian Oscillators

While rhythms in physiology and behavior are often studied at the whole-organism or tissue level, the underlying circadian oscillatory machinery can reside within individual cells. Indeed, circadian rhythms have been described in unicellular organisms, including cyanobacteria and in individual neurons isolated from the mammalian SCN (Welsh et al., 1995; Liu et al., 1997). The master clock regulating circadian behavior is located in the suprachiasmatic nucleus of the anterior hypothalamus (Reppert & Weaver, 2002). There are also circadian oscillators in other tissues (Balsalobre et al., 1998; Reppert & Weaver, 2002; Yagita et al., 2000; Yamazaki et al., 2000; Zylka et al., 1998). The same genes involved in the intracellular SCN clock mechanism are rhythmically expressed in other brain areas and in peripheral organs, both *in vivo* and *in vitro*. The SCN synchronizes the timing of extra-SCN ("peripheral" or "slave") oscillators (for review see Schibler et al., 2003). Synchronized slave oscillators, in turn, regulate local (tissue-specific) rhythms in physiology and behavior.

The intracellular, or "cell-autonomous," nature of circadian rhythms is important for our mathematic modeling efforts, because it allows us to model relevant aspects of system behavior by modeling intracellular processes within a single cell. While tissue-level interactions between cells and even between organ systems are important for whole-animal physiology, these interactions are not necessary for oscillatory behavior in neurons or for the construction of valid computational models of the circadian oscillator.

Molecular Basis of the Mammalian Circadian Clock

At the molecular level, circadian oscillations are based on the rhythmic expression of clock genes. The intracellular clock mechanism in the mouse SCN is based primarily on a transcriptional-translational negative feedback loop**.** Molecular elements that comprise this system in mammals have been identified beginning in 1997. (**Format note**: genes are italicized, while the proteins from these genes are in all capital letters).

Two basic helix-loop-helix/PAS-containing transcription factors, CLOCK and BMAL1 (MOP3) provide the basic drive to the system by activating transcription of negative regulators through E box enhancer elements (King et al., 1997; Gekakis et al., 1998). CLOCK:BMAL1 heterodimers drive transcription of three *Period* genes (*mPer1-3*) and two *Cryptochrome* genes (*mCry1-2*) (Reppert & Weaver, 2002). mPER and mCRY proteins form complexes, translocate to the nucleus, and interact with CLOCK:BMAL1 heterodimers to inhibit transcription, closing the

feedback loop (Kume et al., 1999; Lee et al., 2001; van der Horst et al., 1999; Vitaterna et al., 1999). Analysis of mice with targeted disruption of the circadian-relevant genes described above reveals the importance of these genes in circadian rhythmicity (Bae et al., 2001; Bunger et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999; Zheng et al., 2001; see Reppert & Weaver 2002 for review).

A "positive loop" regulates the rhythmic expression of *Bmal1* (Shearman et al., 2000b; Preitner et al., 2002; Ueda et al., 2002). *Bmal1* RNA levels are rhythmic, and the rhythms are in antiphase to those for the *mPer* and *mCry* genes. Rhythmic *Bmal1* transcription is due to rhythmic repression by REV-ERB-alpha (Preitner et al., 2002; Ueda et al., 2002). *Rev-Erb-alpha* RNA is regulated through E box elements, and has a phase of production similar to that of *mPers* and *mCrys.* mPER2 may also play a role in stimulating *Bmal1* expression (Shearman et al., 2000b; Zheng et al., 1999). This positive feedback loop regulating the expression of *Bmal1* appears to be less important than the negative feedback loop in the mammalian system. Studies of REV-ERB-alpha-deficient mice reveal that *Bmal1* RNA levels in the SCN are constantly high and not rhythmic, consistent with the proposal that rhythmically produced REV-ERB-alpha normally acts to repress *Bmal1* transcription (Preitner et al., 2002). Nevertheless, REV-ERB--alpha deficient mice maintain circadian locomotor activity rhythms in constant conditions, indicating that rhythmic *Bmal1* expression is not necessary for circadian clock function.

Mathematical Models of Molecular Circadian Oscillators

When early computational models of the circadian timing system were developed, the underlying molecular mechanisms of the circadian system were not understood. These models thus used a modified Van der Pol equation to model the limit-cycle dynamics of circadian systems without associating the model variables to any particular biological substrate. Beginning in the mid-1990's, information about the molecular basis of circadian rhythms in the fruit fly (*Drosophila melanogaster*) emerged, allowing development of mathematical models with a molecular basis. These *Drosophila* models have been expanded and refined as biological studies have revealed more information about the molecular mechanisms for oscillatory behavior (Goldbeter, 1995; Leloup & Goldbeter, 1998; Leloup & Goldbeter, 1999; Smolen et al., 2001; Smolen et al., 2002; Tyson et al., 1999; Ueda et al., 2001).

The overall structure of the circadian system in *Drosophila* and mammals is similar. In both types of organisms, circadian rhythms are based on an intracellular molecular feedback loop (Reppert & Weaver, 2000). Furthermore, homologs of most of the genes involved in the mammalian circadian clockwork were first identified in the fly circadian clock. While there has been shuffling of functions between the molecular components of the clock between species, and gene duplication in mammals has increased the complexity of the mammalian feedback loops, there is nevertheless important similarity between the *Drosophila* and mammalian clock mechanism. Thus, mathematical models of the *Drosophila* oscillator provided the starting point for our efforts to develop models for the mammalian circadian oscillator. One of the models developed under the period of DARPA support (Leloup and Goldbeter, 2003) is an adaptation of a previous *Drosophila* model developed by the same investigators (see Goldbeter, 2002 for review). A second model (Forger and Peskin, 2003) was based on experimental data from mammals and uses different philosophical as well as different mathematical approaches. A review of our Team activities published in 2003 is included in Appendix 1 (Forger et al., 2003).

Project Objectives and Relationship to DOD Objectives and Interests

The success and effectiveness of DOD missions depend on the ability of military personnel to maintain a high level of cognitive performance and vigilance while operating and monitoring sophisticated instrumentation. However, military personnel commonly experience sleep disruption, together with misalignment of circadian phase during their missions, particularly if the missions involve sustained operations, night operations or trans-meridian travel. These types of conditions are associated with deterioration of neurobehavioral performance, and can result in critical lapses of attention during the extended-duty hours required during DOD missions.

Two interacting processes determine the extent of performance degradation associated with jet lag and sleep deprivation: a circadian process and a sleep-wake dependent process. Kronauer, Jewett and colleagues incorporated these processes into a mathematical model that is able to accurately predict human neurobehavioral performance under a variety of sleep/wake conditions such as those experienced by military personnel. The circadian component of this performance model is based on our extensively-validated mathematical model of the effects of light on the human circadian pacemaker first developed when the underlying molecular mechanisms of the circadian system were not well understood. As noted above, however, there has been tremendous progress in identifying the molecular structure of the circadian clock at the

cellular and molecular level in the past decade. Development of detailed, deterministic models of the intracellular circadian pacemaker would allow the output of a more biologically based model to provide the circadian input to the existing mathematical model of human neurobehavioral performance. Therefore, our research program sought to develop and experimentally validate computational models for the intracellular circadian oscillator. To do this, we conducted experimental studies to further identify and characterize interactions between the key molecular components of the cellular circadian clock, developed two deterministic models of the mammalian circadian oscillator, and began to develop mathematical approaches to model this high-dimensional system in a simpler representation that would allow it to be incorporated into models of the human circadian pacemaker. Funding for our effort was curtailed prior to our taking the important step to link these mathematical models of the circadian oscillator to existing models describing the impact of the circadian clock on the human neurobehavioral performance.

METHODS AND PROCEDURES

The procedures employed by the Experimental Group involve analysis of the levels and characteristics (e.g., intracellular localization and phosphorylation state) of key “clock proteins.” Detailed materials and methods are indicated in the articles resulting from this work. Generally, the methods involve isolation of clock proteins, separation by electrophoresis, and detection by Western blotting using antibodies. In some cases, subcellular fractionation, co-immunoprecipitation, or immunohistochemical localization of proteins was performed. Levels of mRNAs for clock proteins were assessed by ribonuclease protection assay and in situ hybridization. The source of the molecules of interest was either tissues removed from mice at selected time during the 24-hr circadian cycle, or cultured cells. Circadian behavioral studies of mice with targeted disruption of circadian genes was performed by standard methods, in which mice are singly housed in cages equipped with running wheels and their voluntary wheel running is detected by a computer-based system.

Mathematical modeling studies were performed as described in each modeling article in the Appendix.

Software efforts from our Software Group led to submission of several circadian models into the “Sandbox” area of the BioCOMP website. A number of model formats were submitted, with documentation, (see Results).

RESULTS

Results will be presented in thematic areas that relate directly to our published papers. The list of papers published with support from this grant is in Appendix 1.

A. Experimental Group Results

1) Posttranslational Mechanisms in the Circadian Clock (Lee et al., 2001)

While the critical structure of the circadian feedback loop is known to be a transcriptional-translational feedback loop, little was known regarding the proteins and their post-transcriptional modifications. Efforts to characterize circadian protein rhythms have focused on liver tissue, and have required generation of specific antisera for mouse proteins (Lee et al., 2001). These antisera have been used for three types of experiments: Western blot analysis (detection of protein levels and molecular size), immunoprecipitation (isolation of protein complexes by precipitation with an antibody, then identification of co-precipitating proteins by Western blot analysis), and chromatin immunoprecipitation (isolation of chromatin fragments by precipitation with an antibody to protein bound to DNA, followed by assessment of the levels of specific DNA sequences in the precipitated material).

The analysis of circadian protein rhythms revealed several important findings (Lee et al., 2001). Absolute levels of the mCRY proteins are significantly higher than PER levels. Cellular fractionation studies reveal that mCRY is present in the cytoplasm throughout the circadian cycle. Nuclear entry of the mCRY and mPER proteins occurs simultaneously, both in liver and in SCN (Hastings et al., 1999; Lee et al., 2001), suggesting that the highly rhythmic production of mPER proteins is the key to regulating nuclear entry of the complex consisting of the negative regulators (Lee et al., 2001).

An unexpected finding was that the levels of casein kinase I epsilon exceed PER levels throughout the circadian cycle (Lee et al., 2001). This situation indicates that the enzyme is actually more abundant than its substrate.

Consistent with previous work in *Drosophila*, analysis of mouse proteins revealed temporal changes in phosphorylation state of circadian proteins, especially mPER1, mPER2, CLOCK, and BMAL1 (Lee et al., 2001). These changes in phosphorylation state (detected by alterations in electrophoretic mobility) occur coincident with changes in cellular localization and inter-

molecular interactions, indicating an important role for post-translational mechanisms in clock function.

2) Details of Transcriptional Mechanisms (Etchegaray et al., 2003)

The accessibility of DNA for interaction with transcription factors is strongly influenced by the interaction of DNA and histone proteins in nucleosomes. Post-translational alterations in histone H3 and histone H4 (e.g., phosphorylation, acetylation, and methylation) at specific residues in the amino-terminal tail of these proteins are highly correlated with alterations in transcriptional activity of specific genes. In liver, a circadian rhythm in histone modification occurs at the promoters of the *mPer1, mPer2*, and *mCry1* genes (Etchegaray et al., 2003). The acetylation of histone H3 is rhythmic, and the rhythm parallels the rhythms in *mPer1* RNA levels and in RNA polymerase II binding to the promoter. The rhythmic acetylation of histone H3 is likely accompanied by other covalent modifications (e.g., histone phosphorylation and methylation) that collectively alter chromatin structure. Thus, the circadian rhythmicity in transcription appears to be regulated by a rhythm in chromatin remodeling which prepares the promoter region for the activation/inhibition cycle. The mCRY proteins may repress CLOCK:BMAL1-mediated transcription by inducing alterations in chromatin structure (e.g., disrupting a coactivator complex or recruitment of a histone deacetylase activity).

3) Mechanisms of Circadian Entrainment in the SCN

A critical feature of the circadian timing system is the ability to be entrained by the environmental light/dark cycle. Time-dependent responsiveness to light is a characteristic feature of circadian clocks in diverse species. Exposure of mice to light early in the night shifts the clock so that subsequent cycles begin at a later time. Exposure to light late in the night, in contrast, advances the circadian clock.

Several lines of evidence suggest that mPER1 and mPER2 play important roles in mediating phase-shifting responses to light. Our studies of mice with disruption of *mPer* genes indicate, however, that neither mPER1 nor mPER2 is absolutely required for phase-shifting by light (Bae & Weaver, 2003). Results from another group contradicted our findings (Albrecht et al., 2001), but in a subsequent paper this group seems to conclude that *mPer* gene products are not absolutely required for phase shifting responses to light (Spoelstra et al., 2003), in

agreement with our work. These studies suggest that multiple molecular pathways may be activated in response to light, and that effort should be spent on identifying these pathways.

One alternative pathway for light-induced resetting of the circadian clock has been proposed involving rapid, light-induced destruction of BMAL1 protein. (This would be an attractive mechanism, as light-induced degradation of the TIM protein is critical for resetting of the *Drosophila* circadian pacemaker). This proposal is based on immunoblot studies conducted with rat SCN (Tamura et al., 2000). The authors reported a circadian rhythm in BMAL1 protein, with peak levels occurring at night, and rapid degradation of BMAL1 after exposure to light at night. We have recently reexamined this issue in mice. Our study (von Gall et al., 2003) shows that BMAL1 and CLOCK proteins are continuously expressed at high levels in the mouse SCN, supporting the hypothesis that rhythmic negative feedback plays the major role in rhythm generation in the mammalian pacemaker. Furthermore, light exposure did not lead to degradation of BMAL1 protein in the mouse SCN, as assessed by both immunocytochemistry and immunoblot analysis (von Gall et al., 2003). These results indicate that rapid degradation of BMAL1 protein is not a consistent feature of resetting mechanisms in rodents.

4) Experimental Group- Work Not Reported in Publications

We worked extensively with cell lines, with the objective of being able to induce rhythmicity under controlled conditions and also to visualize rhythmicity within individual cells. Specifically, we generated a number of constructs with destabilized fluorescent reporter proteins under control of E-box containing promoter elements. It was expected that the reporter gene would oscillate in vitro after experimental perturbation (e.g., serum shock; see Balsalobre et al., 1998; Yagita et al., 2001). However, we were unsuccessful in generating stable cell lines that show the expected rhythmic expression patterns. During the course of our work other groups were successful in using luciferase as a reporter for monitoring circadian gene expression in vitro, and within the last few months two groups reported success with single-cell imaging (Welsh et al., 2004; Nagoshi et al 2004). The availability of data on the precision of single-cell oscillators from these sources has led us to curtail our experimental efforts in this area. Danny Forger of our Modeling Group has been in contact with Dr. David Welsh and will be able to obtain detailed data on individual oscillatory cells. Almost all existing data addresses the mean behavior of populations of oscillators without addressing the behavior of individual oscillators. Analysis of

oscillations in single cells will impact our understanding of oscillator robustness and the importance of molecular noise.

The Experimental Group also worked rather extensively with stable human cell lines transfected with constructs that would allow inducible expression of mouse circadian proteins (O. Gildemeister, K. Bae, O. Froy and D.R. Weaver, unpublished). We were successful in generating cell lines with inducible expression of mouse PER1, we were unable to detect rhythmic expression of endogenous genes or proteins in these cell lines, or even in the parental, untransfected line, following either serum shock or ponasterone-induced expression of mPER1. It appears that the original cell line chosen for this line of investigation was aberrant in its inability to show circadian rhythms, and thus precludes further study.

A final line of study that is continuing, and should lead to an additional publication, is the analysis of the impact of BMAL1-deficiency on circadian gene expression and circadian protein levels. Our results indicate that the impact of BMAL1-deficiency is tissue-specific, with the SCN being exceptionally dependent upon BMAL1 for maintenance of gene expression levels (von Gall, DeBruyne, Lee, Reppert and Weaver, manuscript in preparation), while in the hippocampus the effects of BMAL1-deficiency are more limited. This study reveals that other mechanisms for regulating circadian genes exist in hippocampus, and models describing circadian gene expression in SCN may not be applicable to other tissues.

B. Modeling Group Results

1) Overview

Prior to the start of the current project, Kronauer, Forger and Jewett developed a robust, thoroughly validated mathematical model that accurately predicts the effects of light on the human circadian pacemaker and incorporated this circadian model into a mathematical model that accurately predicts human neurobehavioral performance under different sleep/wake schedules (Forger et al., 1999; Jewett & Kronauer, 1998; Jewett & Kronauer, 1999; Jewett et al., 1999; Kronauer et al., 1999). One limitation of this human circadian model is that it relies on a modified Van der Pol equation to describe the limit-cycle dynamics of the circadian system, without associating the model's two state variables to any particular biological substrate. In order to overcome this limitation, Forger and Peskin (2003) and independently, Leloup and

Goldbeter (2003) have developed biologically-based computational models of intra-cellular clocks. One of these deterministic mammalian models was developed as an adaptation of a previously existing *Drosophila* model (Leloup & Goldbeter, 2003). A second, more detailed, strictly mammalian model of the mammalian circadian oscillator has been developed based, to the extent possible, on experimental data collected as a part of this research program (Forger & Peskin, 2003).

There are several features that one would expect to be achieved by a circadian model, including a free-running period near 24 hours, synchronization to light-dark cycles (entrainment), and phase-dependent phase shifts in the oscillation. Both models meet the major criteria. Within each model, there are terms for the processes influencing each molecular entity, including: RNA production (transcription) and degradation, intracellular movement of RNA, protein and protein:protein complexes, interactions between proteins and alteration in their localization, stability, and activity as a result of these interactions. The major difference between the two models is the level of biochemical detail they represent.

Subsequent work has led to publication of a stochastic version of the Forger-Peskin model (Forger & Peskin, 2005), and papers exploring the details and predictions of the deterministic models (Leloup & Goldbeter, 2004; Forger & Peskin, 2004).

2) The Leloup-Goldbeter (2003) Mammalian Model

As part of out Team project, Jean-Christophe Leloup and Albert Goldbeter developed a deterministic model of the mammalian circadian pacemaker (Leloup and Goldbeter, 2003). The modeling techniques used are similar to a previous model they developed of the *Drosophila* circadian oscillator (Leloup and Goldbeter, 1998). The Leloup-Goldbeter (2003) mammalian model does not distinguish among the products of the three *mPer* genes or the two *mCry* genes, and instead uses single *mPer* and *mCry* entities (RNA and proteins), and with phosphorylation as a mechanism controlling protein degradation. Enzyme-substrate interactions are modeled with Michaelis-Menten type expressions, and transcription regulation is modeled by Hill-type expressions. The model consists of 16 equations. By the addition of three additional equations and the modification of one other, the model can be modified to explicitly include the negative influence of REV-ERB-alpha on *Bmal1* transcription. Light input to the model is achieved through elevation of *mPer* RNA levels. This model affords the benefits that

there are a limited number of equations and that the model can also be used as a *Drosophila* model simply by renaming variables and changing the effects of light.

3) Leloup and Goldbeter (2004)

In a second publication (Leloup & Goldbeter, 2004), this computational model of the mammalian circadian clock was extended to perform sensitivity analysis and investigate entrainment mechanisms. By using different sets of parameter values producing circadian oscillations, the effect of the various parameters was compared revealing that both the occurrence and the period of the oscillations are generally most sensitive to parameters related to synthesis or degradation of *Bmal1* mRNA and BMAL1 protein. (Notably, in the Forger-Peskin model, below, the *Bmal1*-related components are constitutive).

In this model, as in others, the mechanism of circadian oscillations relies on the formation of an inactive complex between PER and CRY and their activators CLOCK and BMAL1. Bifurcation diagrams and computer simulations unexpectedly indicate the possible existence of a second source of oscillatory behavior arising solely from the negative autoregulation of *Bmal1* expression.

When incorporating light-induced expression of the *Per* gene, the model accounts for entrainment by light-dark (LD) cycles. Long-term suppression of circadian oscillations by a single light pulse can occur in the model when a stable steady state coexists with a stable limit cycle. The phase of the oscillations in LD depends on the parameters that govern the level of CRY protein, with small changes in CRY levels shifting the peak of *Per* mRNA from the light to the dark phase. Further changes in CRY levels can prevent entrainment.

4) The Forger-Peskin (2003) Mammalian Model.

Recent data on the mammalian circadian oscillator (described above) provide a detailed view of transcription regulation, as well as post-translational events, including phosphorylation. To model these data, Forger and Peskin felt that the actual binding between kinases and substrates must be used instead of Michaelis-Menten dynamics. In addition, data from the Experimental Group provides a more detailed picture of transcriptional regulation than Hill-type expressions allows. Thus, Daniel Forger and Charles Peskin developed a new mammalian model (Forger and Peskin, 2003) which considers these details.

The Forger-Peskin (2003) model incorporates a series of 73 equations. The molecular entities tracked in the model include: mPER1, mPER2, mCRY1, mCRY2, and casein kinase I proteins, and their corresponding mRNAs. CLOCK and BMAL1 are constitutively expressed (while in the Leloup-Goldbeter model, rhythmicity of these molecules is critical for rhythmicity of the system; see Leloup and Goldbeter, 2004). PER3 is not included in the model, as it appears not to play a role in the core circadian feedback loop (Shearman et al., 2000a; Bae et al., 2001). The model considers multiple phosphorylation states of several of these proteins where experimental data indicates this is the case. Incorporating this level of detail allows us to test the differential roles of mPER1 and mPER2 in phase resetting, simulate mutations in individual proteins (e.g., mPER1 or mPER2), and study specific aspects of phosphorylation (e.g. the *tau* mutation) or transcription regulation. Entrainment to light-dark cycles is achieved by elevation of *mPer1* RNA levels. This model achieves a good agreement with experimental data.

5) Forger and Peskin, 2004

In a recent paper, Forger and Peskin explored features of their 2003 model more fully (Forger and Peskin, 2004). They used a systematic parameter fitting procedure to explore model behavior in the face of perturbations and to define critical features for oscillatory behavior. Based on these simulations, an important role for regulation of *mPer* RNA stability is postulated. The study also indicates tissue differences in the role of REV-ERB-alpha in regulating CRY, with the SCN being relatively immune to the impact of REV-ERB-alpha while in the liver higher REV-ERB-alpha levels play an important role in controlling the peak of CRY levels. Introducing rhythmicity into the CLOCK:BMAL1 complex leads to 12-hour (non-circadian) rhythmicity, suggesting that constitutive expression of this complex is functionally important in the SCN.

As with the Leloup-Goldbeter mammalian model, the Forger-Peskin model behaves as a limit cycle oscillator. In both models, perturbations can bring the model close to the phaseless point, a stable steady state from which oscillations show slow amplitude growth until recovery. Notably, the relaxation in this model takes place on a nearly 2-dimensional manifold, consistent with van der Pol type behavior (Forger and Peskin, 2004). This behavior and the two-dimensional manifold representation are important for subsequent work to mathematically simplify this complex model using two-dimensional manifolds (Forger & Kronauer, 2002; see

also below, discussion of Indic et al.), e.g., so that it can serve as an input to the human neurobehavioral model.

6) Forger and Peskin (2005): A Stochastic Version of the Forger-Peskin (2003) Model

Circadian clocks are remarkably accurate at timing biological events despite the randomness of their biochemical reactions, particularly when reactant concentrations are low. In this paper, Forger and Peskin (2005) examined the causes of this immunity to molecular noise in the context of a detailed stochastic mathematical model of the mammalian circadian clock. This stochastic model is a direct generalization of the deterministic mammalian circadian clock model previously developed (Forger & Peskin, 2003; see above and Appendix 1). A feature of the Forger-Peskin (2003) model is that it completely specifies all molecular reactions, leaving no ambiguity in the formulation of a stochastic version of the model.

With parameters based on experimental data concerning clock protein concentrations within a cell, based in large part on results generated from our Experimental Group, this model finds accurate circadian rhythms occur only when promoter interaction occurs on the time scale of seconds. Thus, there is counterintuitive dependence of long-period (circadian) rhythms on very fast kinetics of promoter interaction. The deterministic version of the model did not reveal this highly dynamic nature of promoter occupation because it considers only the average behavior of an ensemble of promoters, rather than the activation of individual promoters within each cell. These findings have important implications for experimental studies assessing the histone modifications coupled with promoter activation and inactivation and will spur further study. Additionally, a study by Barkai & Leibler (2000) suggesting that circadian oscillations would be noisy and unstable when molecular concentrations were low may now be reconciled with other work; low concentrations of circadian proteins can maintain accurate oscillations if the individual events on the promoter occur rapidly (see also Gonze et al., 2002; Gonze et al., 2003).

As the model is scaled up by proportionally increasing the numbers of molecules of all species and the reaction rates with the promoter, the observed variability scales as $1/n^{0.5}$, where n is the number of molecules of any species.

To assess the mechanisms for robust and accurate rhythmicity while relatively few molecules are involved in the feedback loops, the effects of removing individual gene products was examined. The results show that having multiple members of a gene family (arising from gene duplication) increases robustness by providing more promoters with which the

transcription factors can interact. The model predictions for mPER2 mutants differ between the stochastic and deterministic versions of the model; while mPER2 mutants were not rhythmic in the deterministic version of this model, they are rhythmic in the stochastic version. Additional analysis indicates that stochastic treatments of a similarly structured model may have greater tendency to oscillate than the deterministic version.

7) Towards Incorporation of an Intracellular Model into Human Performance Models

A major remaining objective is the mathematical reduction of an intracellular mammalian model to allow its integration as an input component to an existing model of the human circadian pacemaker and its role in regulating neurobehavioral performance. More specifically, a model exists describing the influence of light on the human circadian pacemaker and on performance (Forger et al., 1999; Jewett & Kronauer, 1998; Jewett & Kronauer, 1999; Jewett et al., 1999; Kronauer et al., 1999). This mathematical model (developed before our period of DARPA support) accurately predicts human neurobehavioral performance given previous light exposure and sleep/wake history as inputs. This neurobehavioral model uses a modified Van der Pol oscillator to represent the human circadian pacemaker. Although this model is able to accurately predict the effects of light on the pacemaker in a wide variety of conditions, it is limited in that its variables are theoretical rather than being biologically based. Therefore, effort within our DARPA-funded project was aimed at replacing the circadian pacemaker component of the neurobehavioral models with an intracellular circadian model. (One of the proposed aims related to this effort was cut upon initial funding of our Project, and the project period was curtailed before we could complete the proposed work.)

Representing the greater biological detail embodied in the intracellular circadian models consisting of many mathematical equations poses great challenge for the analysis of such systems and increase the computation time for solving these equations. Development of a method that retains the predominant dynamics while still providing biologically detailed information is imperative. Two high-dimension mathematical models of intracellular mammalian model have been published by our Team (Leloup and Goldbeter, 2003; Forger and Peskin 2003). Each of these high dimension models was projected onto a manifold using Proper Orthogonal Functions obtained from the empirical decomposition of a model's phase space to obtain a two-dimension model (Indic, Gurdziel, Kronauer and Klerman, manuscript in preparation). The resulting two-dimension representation of each model predicts most of the

salient characteristics of a biological clock including ~24 hr oscillations, entrainment to a LD cycle, and amplitude recovery dynamics that emerge following amplitude suppression. With further refinement and analysis, this approach would have allowed us to incorporate the intracellular model into existing current models of human performance, the output of which is of key importance to military applications.

C. Software Group Cumulative Report

Our Software Group's submissions to the BioCOMP website provide resources to allow the BioSpice community and the public to work with published circadian oscillator models. These models have been submitted in several formats, primarily Matlab and SBML. The submissions include parameter sets and documentation. The following models of the *Drosophila* intracellular circadian oscillator have been submitted: Goldbeter, 1995; Leloup and Goldbeter, 1998; Leloup and Goldbeter, 1999; Ueda et al., 2001. In addition, the Software group has also submitted the two deterministic models of the mammalian circadian oscillator developed within this project (Forger and Peskin, 2003; Leloup and Goldbeter, 2003).

Circadian Performance Simulation Software (CPSS) was developed with support from other agencies by the Biomathematical Modeling Unit at Brigham & Women's Hospital, including members of our Modeling and Software Teams. This software embodies the existing human Neurobehavioral performance model based on a van der Pol oscillator. For any light-dark/sleep-wake cycle input, the software provides predictions of circadian phase, cognitive throughput performance and the subject's subjective alertness. This software has also been provided as a courtesy to several DARPA-funded investigators and DOD labs.

During this DARPA-funded project period, the Software Group provided important support for model development. Effort was placed in development of user interfaces for the models. The Software Group was also instrumental in efforts to reduce the multidimensional models of the circadian oscillator in preparation for using these models as the circadian component of the human neurobehavioral model (see Modeling Group report, above). The Software Group made a significant investment to assess BioCOMP resources potentially useful for model development, such as assessing tools for automatic parameter fitting and for determining the quality of fit for objective comparison of model predictions with experimental results.

DISCUSSION

The circadian oscillator represents a useful test case for the principle underlying the BioCOMP program. A relatively circumscribed set of molecular and genetic interactions within individual cells leads to a robust biological event, oscillation, with a period of approximately 24 hours. This system is thus amenable to approaches to describe it through computational models that capture the essence of the molecular and biochemical details. Thus, circadian biologists have a cellular model system for experimental analysis and mathematical modeling, with tangible implications for human health, performance and "mission readiness".

The intracellular circadian oscillation underlies oscillations that occur at the level of physiology and behavior, and these physiological oscillations have important functional implications. Fatigue, sleep loss and circadian factors are major components in the "human factors" that interfere with the ability of military personnel to maintain cognitive performance and vigilance at high level for long intervals. The circadian timing system plays a major role in determining the quality and timing of sleep as well. There is also an increased incidence of stress-related illnesses in individuals that work shifts or fight their bodies to stay awake, so the health of military personnel performing even routine, non-battlefield functions can be affected by the demands of a 24/7 staffing schedule. Optimization of work schedules through attention to circadian principles is increasingly common in the private sector workplace. Timed light exposure offers significant advantages over pharmacological approaches (e.g., alertness-promoting stimulants), to optimize performance and mission readiness, but the understanding of the effects of light on the human circadian pacemaker is relatively primitive. Increased understanding of the circadian oscillator from a biochemical and mathematical perspective should yield methods to manipulate its phase and amplitude, providing improved pharmacological strategies to either "stop the clock" or re-set it with precision, regardless of ambient lighting conditions. Control over the circadian oscillator should thus promote adaptation to shift work and non-24 hour duty cycles, and minimize the physiological disturbance caused by shift work, jet lag, and extended duty periods.

CONCLUSIONS

This project brought together for the first time a group of scientists dedicated to using experimental and computational methods as complementary and interactive approaches to understand the mammalian circadian oscillator. Our Experimental Group had considerable success in gathering information on biological parameters and in identifying the molecular mechanisms of rhythmicity. Our Modeling Team developed two completely independent models of the mammalian circadian oscillator. Both models meet the expected criteria for an intracellular circadian model, with near-24 hour cycle length of oscillations in constant conditions and entrainment to light-dark cycles. While a strict comparison of the two models has not been conducted, it is clear that several key assumptions and resulting predictions differ between these two models. Methods used to deal with the complexity of these models led to innovative strategies for model reduction. Through our publications in the scientific literature and the availability of software submitted through the BioSpice website, our Team's efforts will make an important contribution to the BioCOMP Program's efforts to model cellular processes by providing information on the ubiquitous process of circadian oscillation.

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APPENDIX 1: List of Publications supported by DARPA contract F30602-01-2-0554
"Development and Validation of a Computational Model for Intra-Cellular Circadian Oscillators"

Bae K, Weaver DR (2003). Light-induced phase shifts in mice lacking mPER1 or mPER2. *J Biol Rhythms* 18: 123-133.

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Manuscripts In Preparation (not included- manuscripts will be forwarded when published).

Indic P, Gurdziel K, Kronauer RE, Klerman EB. Development of a two-dimension manifold to represent high dimension mathematical models of the intracellular mammalian circadian clock. *Manuscript in preparation.*

von Gall C, DeBruyne JD, Lee C, Reppert SM, Weaver DR. Tissue-specific regulation of mPER proteins in BMAL1-deficient mice. *Manuscript in preparation.*